''Control of Ciliate Protozoa in the Rumen by Using a Mixture of Saponin and Stevia Extracts''

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25th September, 2015

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ACKNOWLEDGEMENTS

My sincere thanks goes to Prof. Jamie Newbold for his professional guidance and support during this dissertation.

I would like to thank Hilary Worgan and especially Dr. Eva Ramos Morales for her assistance and guidance during the project work including the laboratory and field work.

And finally, I would like to acknowledge the support of my fellowship the Republic of Turkey Ministry of National Education and Ministry of Food, Agriculture and Livestock which funded my MSc in Livestock Science.

I must also thank my family back home –Kurdistan- all over the year, for their support, encouragement and quiet patience, all time wishing best and do pray for me. And also friends, especially Farid, for all of their love and support at background.

ABBREVIATIONS

ADF	Acid detergent fiber
ANOVA	Analysis of variance
$BW^{0.75}$	Metabolic body weight
CH_4	Methane
CP	Crude protein
CTAB	Cetyl trimethyl ammonium bromide
DM	Dry matter
DMDP	2,5- Dihydroxymethyl -3,4- dihydroxypyrrolidine
DMI	Dry matter intake
EDTA	Ethylene diamine tetra acetic acid
g/d; l/d	Gram per day; Liter per day
IBERS	Institute of Biological, Environmental and Rural Science
N	Nitrogen
*N (*italic*)*	Normal
NDF	Neutral detergent fiber
OM	Organic matter
PSMs	Plant secondary metabolites
RUSITEC	Rumen simulation technique
SED	Standard error of the difference
VFA	Volatile fatty acid

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''Control of Ciliate Protozoa in the Rumen by Using a Mixture of Saponin and Stevia Extrcats''

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ABSTRACT

Saponins are plant secondary metabolites and have been reported in a variety of plant families. Producing foam in water, saponins name is derived from the Latin word of 'sapo' which means soap. In consequence of poorly being absorbed in intestine, saponins main effects occur in the gut or rumen only. Many studies have reported the effect of saponins on ruminants both *in vivo* and *in vitro* such as removal of protozoa from the rumen microbial system and also manipulation of the end products of fermentation. Engulfment and degradation of bacteria by ciliate protozoa in the rumen significantly reduced microbial protein flow from the rumen by causing rapid intra-rumen nitrogen cycling and then excreting excess ammonia in the urine. In this case the presence of protozoa is undesirable in the rumen. Saponins kill or damage protozoa via forming complexes with sterols in the protozoal membrane surface which cause impaired membrane and finally disintegration. Saponins have been used in many studies to show the methane mitigation in livestock. Saponins are safe, economical, and effective strategy which may decrease this potent greenhouse gas and also, may eliminate loss of ingested feed energy for productive purposes.

In the present experiment eight rumen cannulated sheep, fed a diet balanced to meet maintenance requirements, have been used. The study builds on previous experiments carried out and aims to build on data to confirm the effect of saponins in sheep which kill or damage protozoa.

The aim of this study is to assess the effect of Ivy and Stevia extracts, either on their own or combined, on rumen fermentation in cannulated sheep. The experiment focused on the use of a saponin containing diet (Ivy) to improve nitrogen utilization and mitigation of the methane production by targeting protozoa and combining in the diet with a glucosidase inhibitor (Stevia, DMDP; 2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine) which subsequently, protects the saponins from degradation in the rumen flora. Treatments were; control (no addition of supplement), Ivy (10 g/animal/day), Stevia (20 g/animal/day) and Ivy+ Stevia (basal diet with 10 g/animal/day of the Ivy extract and 20 g/animal/day of the Stevia extract) mixture.

The results have not shown any significant changes ($P>0.05$) based on the apparent digestibility of nutrients, metabolic weight, N balance and methane production in Ivy, Stevia nor Ivy+ Stevia diets comparing to the control group.

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1. INTRODUCTION

1.1 General introduction

Due to the increasing human world population, rapid urbanization and income increases which leads to the higher consumption; the demand to the livestock products increases for their high quality nutritive ingredients, especially in developing countries for example China, India and most African countries. Livestock is also a livelihood for most of the rural people both in developing and developed countries (Thornton *et al.,* 2007). The ruminants can be counted as a crucial contributor to the global supply of human-edible food, owing to the rumen microbial environment which is able to transform fibrous feedstuffs are not ready to be utilized by monogastrics, including human. However, it has been showed that the climate change impacts of livestock production cannot be underestimated because of producing carbon dioxide, methane and nitrous oxide. 18 % of the total global greenhouse gas emissions from human sources are caused by the livestock (Steinfeld *et al.,* 2006).

Methanogenesis caused by the ruminants is thought to be between 13-19 % of all methane emission to the atmosphere and up to 25 % of a total anthropogenic sources (Lowe, 2006). This methane cost to the livestock, especially ruminants, by causing 2-12 % of gross energy intake (Johnson and Johnson, 1995). In recent years, parallel to the growing concern about global climate change, antimicrobial compounds are added to the ruminant diets aiming suppressed methanogenesis, improved animal efficiency, reduced excretion of N in urine and faeces. But unexpected reports of increased antibiotic resistance among pathogenic microorganisms (Carro

and Ranilla, 2003) changed the researchers' attention towards the safer natural products against chemical substances to manipulate rumen microbiota and fermentation (Pen *et al.,* 2007).

1.2 Review of the literature

1.2.1 Rumen microbiota and Protozoa

Rumen microbial ecosystem is extremely diverse and includes bacteria, fungi, protozoa, and archaea. These microorganisms generally perform a symbiotic relationships with their hosts and carry out essential metabolic functions for ruminants' development, health and nutritive values (Morgavi *et al.,* 2010). This microbial community inhabits the anaerobic and methanogenic environment of the rumen where CO_2 and H_2 are produced from the fermentation of feeds by fermenter microbes. Organic matter in the rumen is digested to their monomers and then further to the volatile fatty acids (VFA), CO_2 and H_2 by the primary and secondary fermenters (Edwards, 2004; Yu *et al.*, 2006).

Although, methanogens contribute to the greenhouse gas emission by producing methane, they decrease H_2 level which high level may inhibit function of microbial enzymes involve in oxidation/ reduction reactions. For example, inhibition of NADH dehydrogenase lead to the accumulation in NADH and eventually in reduced rumen fermentation (Morgavi *et al.,* 2010). This process, producing H_2 by one microbial species and capturing by another microbial species, is named as interspecies H_2 transfer (Wolin *et al.,* 1997).

Even though, protozoal population form a large portion of the rumen biomass, still essential arguments take place to clarify their role in rumen fermentation and their contribution to the

metabolism and nutrition of the rumen. Flagella and ciliate protozoan have been described in the rumen. In the rumen more than 250 species of ciliate protozoa have been identified which mainly belongs to the two essential subgroups; the Entodiniomorphida and the Vestibuliferida (**Figure 1**; Williams and Coleman, 1992).

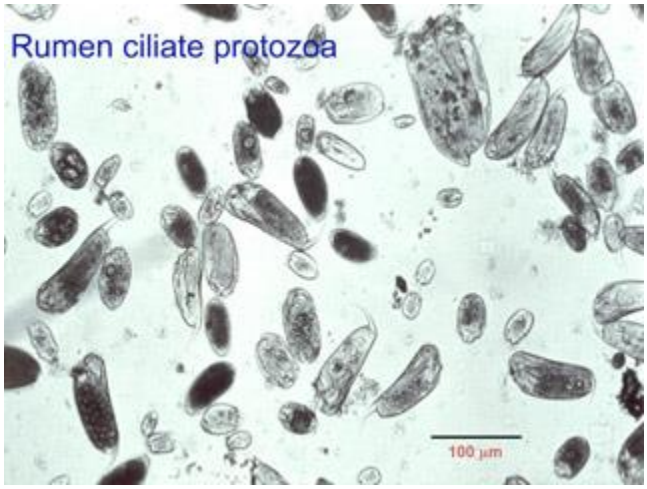


Figure 1 Rumen ciliate protozoa are large enough that they can be seen by naked eye. Engulfing bacteria at a huge rate, protozoa lead wasteful N retention in ruminant, sheep (Wallace, Knowledge Scotland).

Ciliated protozoa in the rumen, with their very active metabolism, capable to influence other microbial populations and fermentation and eventually the end products of fermentation including methane (Williams and Coleman, 1992; Eugene et al., 2004). They engulf organic matter, especially bacteria, and passing to their digestive vacuoles leading to the hydrolysis and fermentation. They have acetate and butyrate as a main VFAs, end products (Williams and Coleman, 1992; Hillman *et al.* 1995). Despite the fact that protozoa are not essential for an animal to survive, but defaunation (removing protozoa from the rumen by applying different chemical

or physical techniques) cause major changes in the rumen microbial population. Defaunation of ruminants from protozoa has been intensively used to define the effects and roles of protozoa in manipulation of rumen microbial population and rumen functions especially fermentation parameters (Williams and Coleman, 1992).

The study builds on previous work carried out and aims to build on data to confirm the effect of saponins in ruminants (sheep) by killing or damaging protozoa populations in rumen microbial system. Morgavi *et al.* (2010) have summarized published *in vivo* trials and showed the effect of defaunation as an average of overall studies by 10.5 % decrease in methane emission (see Appendix 2). In spite of average decrease for overall published studies, a great of variability clearly visible within the available data, even some studies measured increased methane emission when animal defaunated (see Appendix 2). Besides some possible explanations to these result varieties by different methodologies for instance; the method used for defaunation animals, the dietary effect etc. Further researches is required to enlighten the effect of defaunation animals on methanogenesis (Morgavi *et al.* 2010).

Engulfment and degradation of bacteria, fungal zoospores and archaea by ciliate protozoa in the rumen significantly reduced microbial protein flow from the rumen by causing rapid intrarumen nitrogen cycling and then excreting of excess ammonia in the urine (Wallace and McPherson, 1987; Coleman, 1988). This rapid protein turnover leads to decreasing of the supply of bacterial protein to the host organism. In this case the presence of protozoa is undesirable in the rumen.

1.2.2 Methane production and methanogenesis associated with protozoa

Methane is produced by the end products of the anaerobic fermentation, CO_2 and H_2. Some essential methanogens in the rumen live in a symbiotic association with the rumen protozoa which are responsible for up to 37 % of rumen methanogenesis (Finlay *et al.,* 1994). In addition, methanogenesis is assumed to cause 2-12 % loss of energy intake in the ruminants (Morgavi *et al.,* 2010). The estimated amount of methanogens in protozoan cell is 10^3-10^4 before feeding and one to ten after feeding (Tokura *et al.*, 1997). The protozoa in the rumen has ecto-symbiotic and/or endo-symbiotic relationship with methanogens in the rumen. So, it is thought that reducing protozoa will also reduce these methanogens and eventually decreasing methane production (Newbold *et al.,* 1995; Hess *et al.*, 2003).

In defaunated ruminants (sheep) free from ciliate protozoa, methane production is decreased by up to 25 % and microbial protein supply increases up to 50 % (Yanez-Ruiz *et al*., 2007; Morgavi *et al.*, 2008). Sharp *et al.* (1998) have reported a decline in the most abundant archaeal methanogen (*Methanobacteriaceae*) from 84 % to 54 % in fermenters with the loss of protozoa. They also has showed in protozoa washed out rumen that some methanogens (*Methanomcrobiales*) increased which might be because of symbiotic relations with protozoa, but some did not (*Methanosarcinales*) due to not having symbiotic relationships with protozoa. Therefore, defaunation of ciliate protozoa from the rumen of sheep should lead to an increased production efficiency and sustainability of domestically supplied sheep meat and milk for food, and also should lead to reducing greenhouse gas, methane, emissions from the supply chain.

Methane emission suppression has occurred in RUSITEC (rumen simulation technique) system by the supplementation of *S. saponaria* or by addition of tea saponin to *in vitro* rumen fermentation (Hess *et al.,* 2003), also the same suppression has achieved by feeding sheep with a *S. saponaria* fruit (Hess *et al.,* 2004).

Although, the direct effect of saponins on methanogens has not been verified constantly, some studies have reported that saponins may reduce the rate of methanogenesis; by decreasing the activity of methane producing genes (for instance *mcrA*) or rate of methane production per methanogenic cell, without changing the total methanogen population (Hess *et al*., 2003; Guo *et al.*, 2008).

Saponins may decrease methane production due to increasing the proportion of propionate from VFAs which channelling hydrogen from methanogenesis to propionate production. On the other hand, increased bacterial and fungal populations by protozoan defaunation, leads greater digestion of feeds and fibre components, so the increased methane production (Goestch and Owens, 1985; Valdez *et al.*, 1986; Pen *et al.*, 2007). In this case, clearly it can be seen that physiological effects of saponins weaker compared with the microbiological effects (Lu and Jorgensen, 1987; Klita *et al.*, 1996).

1.2.3 Saponins and saponin containing plants

In recent years, increasing concerns against chemical residues in animal derived foods and high threats of antibiotic resistant bacteria, has led researchers to explore safer alternatives, plant secondary metabolites (PSMs), such as saponins, condensed tannins and essential oils. These

natural additives have been used to improve rumen metabolism and increase the efficiency of ruminant production (Wallace *et al*., 2002; Wallace, 2004; Benchaar *et al.*, 2008; Kamra *et al*., 2008).

Saponins are PSMs and have been reported in a variety of plant families; some plants widely applied for high saponin contents are *Quillaja saponaria, Medicago sativa (*alfalfa*), Saponaria officinalis, Hedera Helix (*Ivy plant*), Yucca schifigera* etc. Producing foam in water, saponins name is derived from the Latin word of 'sapo' which means soap (Sirohi *et al*., 2014). Chemically, saponins are either steroid or triterpene glycoside compounds (**Figure 2**). In consequence of poorly being absorbed in intestine, because of their large molecular mass (>500 Da), high hydrogen bonding capacity and molecular flexibility, saponins main effect is occur in the gut or rumen only (Flaoyen *et al*., 2002).

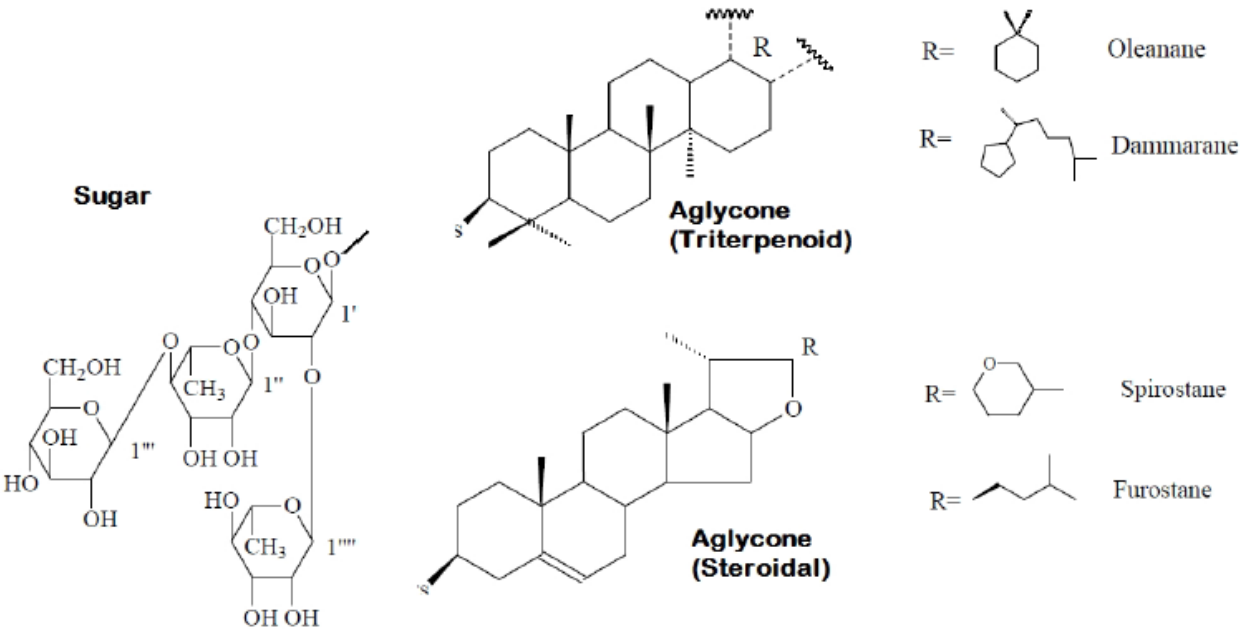


Figure 2. Components and chemical structure of saponins (Sirohi *et al.*, 2014)

Saponins have a wide range of biological activities including antibacterial, antiviral, antifungal, haemolytic, cytotoxic, anti-inflammatory, insecticidal, anti-oedematous, anticancer, antitumor, molluscicidal, piscidal and immunomodulatory action (Hostettmann and Marston, 1995; Sparg *et al.,* 2004; Fuchs, *et al*., 2009; Podolak, *et al*., 2010).

1.2.4 Saponin containing plants' effect on protozoa

Saponins kill or damage protozoa via forming complexes with sterols in the protozoal membrane surface which cause impaired membrane and finally disintegration (Wallace *et al*., 1994; Wallace *et al.*, 2002).

It, also, has been showed that the protozoal counts in sheep (Ivan *et al*., 2004) and Holstein cow (Rosales *et al.*, 1989) rumen fed with *E. cyclocarpum* (saponin containing forage species) decreased without changing the composition of protozoa community. Wina *et al*. (2005) in their review compared *in vivo* (sheep/cattle) and *in vitro* results on the effect of saponins or a variety of saponin containing plants on protozoa in the rumen. The obvious effect of saponins on protozoa could be depend on the dosage, saponin contained plants, animals (sheep, cattle, buffalo), and additional substrates/ feeds (**Appendix 1**). The effect of saponins on protozoa has been measured by looking at the percent decrease in protozoal count or percent decrease in protozoal activity, based on the amount of released [^{14}C] from labeled bacteria. None of these in vivo nor in vitro studies have demonstrated any long term antiprotozoal effect, since the antiprotozoal activity of saponins is transient and the population recover back with a rapid adaptation to saponin (from several days to weeks) (Newbold *et al.,* 1997; Odenya *et al.,* 1997; Ivan *et al.,* 2004). Wang *et al.* (2000) have reported an increase in the thickness of microbes (*P. bryantii*) cell wall in pure culture after adapted to Yucca saponins. In addition, the high glycosidase activity, produced by *Ruminococci*, have reported that more likely looks as a part of the adaptation process. Having previous exposure also may enhance the rapid and quick reaction to the presence of saponins and lessening the antiprotozoal effect of saponin containing plants. Even different sheep species or breed or the animals' environment may affect the ability of rumen microbes, reducing the antiprotozoal activity of saponins (Teferedegne *et al.,* 1999). Therefore, it may not be that much easy to specify the exact mechanism of lessening antiprotozoal activity of microbes to saponins.

Both feed degradation and microbial lysis produce ammonia and some is absorbed by the host animal and the rest utilized by microbes (Wina *et al.,* 2005). Rumen microbes utilize most of their N requirement (50-80%) from the ruminal ammonia-N pool (Leng and Nolan, 1984). So, the decline in the rumen ammonia results in two possibilities; a reduced substrate degradation or to the utilization of ammonia by bacteria (Wina *et al.,* 2005). Protozoa population in the ruminant contributes to the total rumen nitrogen between 10 % and 40 %, so they could have a direct effect on concentration of rumen ammonia. But saponins have an indirect effect on the concentration of the rumen ammonia, since saponins decrease protozoa which means less microbial (bacterial) lysis, then less release of the products of protein breakdown. (Van Soest, 1994).

1.2.5 Transitory effect of saponin on protozoa and alternative aspects

Saponins have a transitory effect on protozoa in the rumen. Different approaches have been developed to tackle this problem; such as feeding animal with a saponin in a diet intermittently which may avoid rapid microbial adaptation (Newbold *et al.*, 1997). The anti-protozoal effect is not driven by protozoa itself but other rumen microorganisms, bacteria, which start to degrade saponins by cleavage of the glycosidic bonds (Newbold *et al.*, 1997; Teferdegne *et al.*, 1999).

Glycosidase inhibitor DMDP, a polyhydroxylated alkaloid (2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine) is another alternative approach to deal with a transitory effect of saponins in the rumen. It has been shown that when DMDP has been given to the ruminant, the acetate/ propionate ratio and the ammonia concentration at 24 hour of incubation decreased in a greater extent than that observed only with the ivy extract. Besides a recovery has been observed at 24

h in all cases, protozoa motility has decreased over time in the Ivy extract and DMDP diet when compared with that of the Ivy extract alone (unpublished data, IBERS).

Due to the high cost of the extraction process of DMDP, the Stevia has been used extract which is rich in DMDP and iminosugars (analog of sugars where a nitrogen atom has replaced the oxygen atom in the ring of the structure). Stevia (*Stevia rebaudiana*) is a perennial plant and commercially used as a sweetener. Its sweetening feature comes from glycosides and their derivatives (Gardana *et al*., 2003; Geuns 2004). Besides its sweetening features it includes 16 % CP and 2.6 % fat in its leaves (Atteh *et al.*, 2011).

The *In vitro* study also showed the reduced acetate/ propionate ratio, ammonia concentration and protozoa motility in the incubation of 2 mg/ml of Stevia extract on its own at 24 h, in comparison with the control one. But the effect of 1mg/ml of the Ivy extract has been recorded stronger than that of the Stevia extract on its own. On the other side, when incubating both extracts together, the decrease in acetate/ propionate ratio and ammonia concentration at 24 h was higher than that caused by the Ivy extract alone. In addition the decline has been observed for the protozoa motility at 8 and 24 h of the incubation, when the Stevia and Ivy extracts were combined in comparison with the Ivy extract alone (unpublished data, IBERS).

Therefore, it can be concluded by the enlightening of the previous studies that Stevia extract could be used as a modulator of the fermentation and rumen protozoa on its own. Another conclusion is that there might be a synergistic effect when combined the Ivy and Stevia extracts. This synergetic effect could be due to the interaction between the saponins and DMDP as well as iminosugars present in Stevia.

1.2.6 Saponins effect on rumen nutrient digestibility

From the positive side; it has been shown that the presence of protozoa in the rumen stabilized rumen pH and decreased the redox potential of rumen digesta and ultimately should stimulate the cellulolytic bacterial activity indirectly (Russell and Wilson, 1996). In addition, one-fifth of fiber degradation is of protozoal origin (Dijkstra and Tamminga, 1995).

Looking from the other side; engulfment of different microbial species by the protozoa leads to a considerable proportion of microbial turnover in the rumen and subsequently decreasing the efficiency of protein utilization in ruminants (Wallace and McPherson, 1987). Therefore, it has been concluded that defaunation will lead to increased bacterial and fungal populations and occupy the flora previously filled by the protozoa (Williams and Coleman, 1997) so, the efficiency of microbial protein synthesis will increase and protein flow to the duodenum and also N retention in animals (Santoso *et al.,* 2007). It has not been founded any significant differences between partially and completely defaunated sheep (Veira *et al.,* 1983) which suggest that the effect of saponins effect on microbial protein synthesis efficiency and flow of amino acids to the intestine might be between complete and partial defaunated ones (Patra and Saxena, 2009).

For decreased ammonia concentration in the rumen, due to using saponins; several aspects have been claimed by different researchers and studies such as; primarily reduction in bacterial lysis because of anti-protozoal effect of saponins; direct depression of the proteolytic and deamination activities which may cause by saponins inhibitory effect on protozoa or directly on bacteria; direct inhibition of microbial urease by saponins (Ellenberger *et al.,* 1985; Patra and Saxena, 2009). In spite of the greater bacterial activities which increasing proteolysis and

deamination, the contribution to the total ammonia in the rumen might be small comparing to the effect of saponins in the diets. So, there will be a logical expectation about different values of ammonia concentration from different studies due to the addition of saponin and its effect level on protozoa and bacteria (Patra and Saxena, 2009).

Although, some studies have demonstrated the decrease in the passage rate of digesta from the rumen (Lu and Jorgensen, 1987) which might has an effect on increasing the ruminal degradation of feeds. But besides the physiological effects of saponins the greater microbiological effects on microbial populations also should be considered. Therefore, some studies might be recorded the positive effects of saponins on the feed digestibility due to the increased bacterial populations, some other might be reported the negative effects of saponins because of the decreased hydrolytic enzyme activities from protozoa or bacteria and fungi affected by saponins (Patra and Saxena, 2009).

Acetate and butyrate are the significant end products of fermentation in protozoa so, logically in defaunated animals a decrease in the acetate/ propionate ratio have been observed and an increase in the proportion of the propionate production (Williams and Coleman, 1997).

Another study has reported that the presence of saponin extracts (Quillaja, *S. rarak*) lead a substantial reduction in the apparent and true digestibility of the substrate in an *in vitro* fermentation (Makkar and Becker, 1996; Wina *et al.,* 2006). In RUSITEC *in vitro* fermentation system, a decrease has been detected for NDF, ADF, and cellulose degradation when the whole fruit of *S. saponaria* was added (Hess *et al.,* 2003). Navas-Camacho *et al.,* (1993) have observed a reduction *in sacco* DM (dry matter) digestibility when *E. cyclocarpum* has been given. Impaired

fiber digestion in the rumen (in vivo) also has been detected when alfalfa saponins have been given (Lu and Jorgensen, 1987). These decreased rumen fiber digestion may be because of the lower fibrolytic enzyme activity in the rumen on account of saponins (Wina *et al.,* 2005) and this supports the fact of excretion of fibrolytic enzymes by protozoa (Williams, and Withers, 1991).

Saponin extracts affect different species of rumen bacteria hence, they may alter different enzyme activities of those species important in metabolism (Wang *et al.,* 1998).

1.2.7 Saponins effect on metabolic weight and N balance

Although, the possible effect of defaunation depends on the energy and protein requirements of animals and nutrients supplied by diets, generally, it is agreed that removing or suppression protozoa would lead to increased ruminant performance (Finlay *et al.,* 1994), especially on a low protein diets and not limited in energy (De Smet *et al.,* 1992; Eugene *et al.,* 2004). Veira *et al*. (1983 and 1984) have reported increased intestinal amino acids availability in the ruminant where partial or completely defaunation from protozoa applied. Therefore, defaunation is a desirable and may lead a higher absorption and utilization of intestinal amino acids by the ruminant (Jouany, 1996; Jouany and Ushida, 1999).

Different studies have reported improvement in saponin containing diets fed sheep; such as increasing in body weight gain in a study (Navas Camacho *et al.,* 1993) and wool growth by 27 % in another study (Leng *et al.,* 1992). As a general observation, from reports mentioned above, it seems that saponins may increase the performance of ruminants of roughage based diet (Patra and Saxena, 2009). In contrast, the same pattern for improving in ruminant performance have

not been reported for concentrate based diets (Hussain and Cheeke, 1995; Zinn *et al.,* 1998; Silwinski *et al.,* 2002; Gorgulu *et al.,* 2004). On account of above studies, saponin containing diets effect on ruminant performance is likely to be diet dependent.

1.3 Gaps in knowledge and scientific hypothesis

The objective of this study is to assess the effect of Ivy and Stevia extracts, either on their own or combined (Ivy+ Stevia), on rumen fermentation in cannulated sheep. In accordance with the current literature it is hypothesized that a diet administered with saponins (Ivy) and a glucosidase inhibitor (Stevia) will be reflected especially in the methane emission and also in balance/ utilization of nitrogen and apparent digestibility of nutrients as well.

Therefore, current experiment has focused on the use of saponin containing diet (Ivy) to improve nitrogen utilization and mitigate methane emission by targeting protozoa and combining diet with a glucosidase inhibitor containing diet (Stevia, DMDP) which subsequently, protects the saponins from degradation in the rumen flora. Saponins will kill/ damage protozoa by forming complexes with sterols in the protozoal membrane surface which leads impaired membrane and finally disintegration. Due to the transient effect of saponins on protozoa a powerful glucosidase inhibitor, DMDP, will be used to avoid deglycosylation and therefore, maintaining the intact saponin and their activity in the rumen.

2. MATERIAL AND METHODS

The experiment was carried out between January and July 2015, at one of the ruminant metabolism research unit of Trawscoed Research Farm, IBERS, Aberystwyth University, UK. All procedures used in this study were licensed and regulated by the UK Home Office under the Animals (Scientific Procedure) Act, 1986. In the present experiment eight barren rumen cannulated sheep, fed a diet balanced to meet maintenance requirements, have been used. This trial has been done in the purpose of assessing the effect of saponins on the methane production and also checking the DM/ OM (Dry matter, Organic matter), nitrogen and digestibility.

2.1 Experimental design

Eight re-faunated sheep were cannulated on 9-11 December 2014 with a surgery (see Appendix 3) and waited till cannulated sheep were recovered (6th of January). Then sheep were fed a restricted diet; 250 g of sugar beet and 1.2 kg of ryegrass hay. The hay were given daily in two periods, in the morning 9:00 a.m. and in the afternoon 5:00 p.m. The animals were weighed at the start and the end of the experiment (**Table 2.1**).

For the present experiment, a replicated 4x4 Latin square design was used with two replications (four treatments, four periods, eight experimental animals and two squares). Each experimental period lasted for 21 days, and followed by two weeks wash out period to refrain the possible carry over effect of the treatments. Ensuring the recovery of the protozoa population after feeding saponins, the eight sheep have been re-inoculated with a mixture of rumen fluid all the sheep, in the first week of the wash out period. For re-inoculation of the sheep between periods

about 200 ml of rumen content were collected from each sheep. These rumen fluid contents were strained through 1 layer of muslin and pooled in thermal flasks. Then, strained rumen fluid was mixed (1:1) with buffer and about 400 ml of diluted mixture were re-inoculated to each sheep via a dose gun.

Table 2.1. Identification of the experimental animals.

N	ID	Sex	Birth	Weaning	BW weaning	BW 23/08/14	BW 4/12/14	BW 9/01/15
1	4440	F	26/03/2012	18/05/2012	17.8	82.0	73.6	74.8
2	4381	F	19/03/2012	09/05/2012	15.8	84.1	81.4	80.0
3	4344	M	15/03/2012	09/05/2012	15.3	85.1	75.8	76.0
4	4367	F	18/03/2012	09/05/2012	14.8	74.4	65.4	70.0
5	4356	M	18/03/2012	09/05/2012	17.8	81.5	77.4	79.4
6	4337	M	14/03/2012	09/05/2012	16.4	81.9	75.4	73.0
7	4421	F	24/03/2012	09/05/2012	15.0	87.3	77.4	80.0
8	43.76	F	19/03/2012	09/05/2012	15.3	79.8	74.8	74.4

To set and allocate diets to the each animal LatinSquare.exe program has been used. The program allocates diets to the animals in a random sequence (**Table 2.2**).

Table 2.2. Experimental design (replicated Latin Square).

Square 1

	Period (column)			
Sheep (row)	1	2	3	4
1	Control	Ivy	Stevia	Ivy+ Stevia
2	Ivy	Control	Ivy+ Stevia	Stevia
3	Stevia	Ivy + Stevia	Ivy	Control
4	Ivy+ Stevia	Stevia	Control	Ivy

Square 2

	Period (column)			
Sheep (row)	1	2	3	4
5	Ivy	Control	Ivy + Stevia	Stevia
6	Ivy + Stevia	Ivy	Stevia	Control
7	Stevia	Ivy + Stevia	Control	Ivy
8	Control	Stevia	Ivy	Ivy + Stevia

Cannulated sheep have been fed with a diet of 1.2 kg of ryegrass hay and 250 g of sugar beet. The experimental diets have been given with the Ivy refined extract and without it, Stevia extract or a combination of both. So, four treatments are; control (just experimental diet), IVY (basal diet including 10 g/animal/day of the Ivy extract), Stevia (basal diet including 20 g/animal/day of the Stevia extract), and Ivy+ Stevia (basal diet with 10 g/animal/day of the Ivy extract and 20 g/animal/day of the Stevia extract). The diet measurements were done based on previous *in vitro* experiments, presuming a rumen content volume of 10 l. During the experiment, sheep were given the concentrate once daily at 9:00 am, and the forage twice a day (9:00 am and 5:00 pm) in the doses of 600 g (1.2 kg total). The Ivy (10 g/animal/day) and Stevia (20 g/animal/day) extracts were given through the cannula just before feeding.

2.2 Animals and housing

The cannulated animals were housed individually, inside barn, in pens with dimensions of 1.70 X 1.50 m, in Trawscoed IBERS farm. Sheep were bedded on shavings that topped up three times weekly (**Figure 3**).

Eight cannulated sheep have been housed, for five days, in individual metabolic crates where placed in the methane chambers for cattle located in Trawscoed IBERS farm. Methane production measurements and nutrient digestibility analysis have been recorded during these five days.

Figure 3 Individual pens for sheep (1.70x1.50 m) in Trawscoed IBERS farm, Aberystwyth University.

At the end of the experimental period the cannulated animals have not been slaughtered but have been used for subsequent experiment.

2.3 Sample collection

At the end of the each experimental period methane production and nutrient digestibility have been evaluated. For this reason, on day 15th to 21st of the experiment cannulated sheep have been housed in individual metabolic crates that were placed in the methane chambers for cattle

in Trawscoed IBERS farm. In addition to the methane, hydrogen, and carbon dioxide; total urine and faeces also have been quantified for three days. By the side, 20 % of the faeces and 10 % of the urine have been collected to evaluate nutrient digestibility, N and energy balances.

To determine the production of methane, hydrogen, and carbon dioxide a MGA 3000 series multi gas analyser has been used and calibrated daily on a known standard gas. As chambers are initially designed for cattle, flow adjustments were necessary. Chamber emissions were corrected for background concentrations of methane. Auto zeroing took place every 12 h with oxygen free nitrogen. Total exhaust gas volume was recorded manually every day by recording the mean airflow using the handheld hot wire anemometer at approximately 9:00 am and 4:00 pm.

2.4 Methane production

To assess methane production, animals were kept in the individual chambers (Trawscoed IBERS farm, Aberystwyth University), with urine and faeces collectors and separators, including hay and sugar beet to supply food and the mineral mixture and always available water. The chambers' dimensions were 3.3 m long X 2.4 m wide X 2.4 m high and a larger version of the small ruminant respiration chambers described in detail by Hart *et al.* (2012).

Methane emissions were measured from each animal individually for three consecutive 24 h periods in open circuit respiration chambers. Chambers were built of powder coated steel frame with a 4 mm polycarbonate shell affixed to it. The chambers were stand on a concrete floor which was covered with a 12 mm rubber mat. The backside of the each chambers had two doors

with an air gap of 33 cm high in the bottom which was not covered by polycarbonate for air passage. A sliding door placed at the front of each chamber facilitated changing the feed bin where a small door let the personnel entering to clean and milk. As a bedding material; sawdust and limestone were used. Two high speed in-line fans placed at the exhaust of each chamber which were ensured the respiration chamber air flow by negative pressure in the system. A stream of ambient air is drawn from the aisle behind the chambers through the air inlet at the back of the chamber. An outlet hole situated on top of each chamber on the front, placed right above the head of sheep, exhausted the air which was circulated naturally within the chamber.

Faeces and urine samples were obtained daily throughout data collection from methane chambers (**Figure 4**). The leftovers also, were weighed, when the samples obtained daily, to consider in the calculation of food supply for animals. The samples (faeces and urine) were placed in plastic bag and boxes, labelled and stored in a freezer (-20 ^{0}C). The animals were kept in the methane chambers seven days (from day 15 to 21) to collect the methane and carbon dioxide production.

Figure 4 Methane chambers for cattle (right) and inside metabolic crates designed for sheep (left) (Trawscoed IBERS farm, Aberystwyth University).

2.5 Nutrient digestibility; Nitrogen, NDF and ADF analysis

For nitrogen analysis; urine, faeces, feed and milk samples analysed by total combustion at 900 ^{0}C and thermal conductivity detection (vario MAX cube, Elemetar, Hanau, Germany). To obtain crude ash values, feed and faeces were analysed by combusting at 550 ^{0}C in a furnace. DM minus crude ash calculation gave the OM values.

To measure NDF the ‘’Neutral Detergent Fiber in Feeds- Filter Bag Technique (for A200 and A2000I)’’ (ANKOM Technology, Macedon, NY, USA) has been applied for feed and faeces samples (Van Soest, 1991). NDF solution consists of; 30.0 g sodium dodecyl sulphate, 18.61 g EDTA (Ethylene diamine tetra acetic acid), 6.81 g sodium borate, 4.56 g disodium hydrogen orthophosphate anhydrous and 10.0 ml triethylene glycol in 1 l deionized water.

For ADF the ''Acid Detergent Fiber in Feeds- Filter Bag Technique (for A200 and A2000I)'' (ANKOM Technology, Macedon, NY, USA) protocol has been applied following NDF analysis (Van Soest, 1991). ADF solution consists of; 20 g cetyl trimethyl ammonium bromide (CTAB) in 1 l 1 *N* sulphuric acid. After being weighed, F58 filter bags (ANKOM Technology, Macedon, NY, USA) have been used to analyse NDF and ADF values of feed and faeces samples. F58 filter bags were soaked in acetone just after analysis, and dried overnight at 60 ^{0}C. In NDF and ADF analysis a correction factor was included for the change in weight of an empty bag in each bag according to the **Equation 1**.

Equation 1 The equation for correction factor in both NDF and ADF analysis.

%NDF/ADF = ((bag and feed weight after – (bag weight before x (blank bag weight after / blank bag weight before)) * 100) / (sample weight before)

2.6 Statistical analysis

Statistical analyses of methane production and nutrient digestibility data have been performed using the GLM procedure of Genstat 15th Edition in a replicated Latin square design with two replicates (rows of different squares are independent but the columns are shared).

Equation 2 Formula used in statistical analyses of methane production and nutrient digestibility.

$Y(i)jkl = \mu + \alpha_{i\ (l)} + \tau j + \beta k + \gamma l + \varepsilon ijkl$

Where:

Y(i)jk = observation

μ = overall mean

$\alpha_{i\ (l)}$ = effect of the *j*-th row (block 1:sheep) that can be different from different squares so they are denoted $\alpha_{i\ (l)}$ for i=1 to p and l= 1 to n.

τj = effect of the j-th treatment

βk = the effect of the k-th column (block 2: period)

γl = effect of the replicate $\gamma 1$ to γn (square)

$\varepsilon(i)jkl$ = residual error.

Table 2.3. Block structure for Repeated measures ANOVA

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Rows	SS_{Row}	$n(p-1)$		
Columns	SS_{Column}	$p-1$		
Replicate	$SS_{Replicate}$	$n-1$		
Treatment	$SS_{Treatment}$	$p-1$	$MS_{Treatment}$	F_0
Error	SS_E	$(p-1)(np-2)$	MS_E	
Total	SS_T	np^2-1		

Data collected for protozoa numbers and fermentation patterns have been analyzed by a repeated-measures ANOVA, using the same blocking structure as above (**Table 2.3**).

In both cases, means among treatments have been compared by least significant difference (LSD) test. Statistical significance were considered if P<0.05.

3. RESULTS

3.1 Chemical composition of hay and sugar beet

The hay and sugar beet, which has been given in all control, Ivy, Stevia and Ivy+ Stevia diets to the cannulated sheep, compositions (g/kg) have been given in the **Table 3.1**.

Table 3.1. Chemical composition (g/kg DM) of the hay and sugar beet comprising the basal diet

	Hay	Sugar beet
Dry matter (g/kg fresh matter; DM)	795	807
Organic matter (OM)	932	878
Crude protein (CP)	62.7	99.8
Neutral detergent fiber (NDF)	683	393
Acid detergent fiber (ADF)	367	171

3.2 Effect of diets on metabolic body weight and N balance

The effect of control, Ivy, Stevia and Ivy+ Stevia diets on cannulated sheep has not shown any statistically significance ($P>0.05$) on metabolic body weight nor N balance. But the effect of these diets variable among different level of N balance (**Table 3.2**), for instance, urine N in Ivy+ Stevia diet has showed lower values comparing to the Ivy or Stevia alone but not to the control diet ($P=0.091$). For nitrogen balance (proportion of N intake); N intake, fecal N, urine N and N

retention results have been showed in the **Table 3.2**, and N utilization as well. The samples were taken from animals during last five days of 21 days trial. For different aspect of N utilization values a variety of equations have been applied such as; digestible N divided by N intake, N retention divided by digestible N and N retention divided by N intake.

Table 3.2. Effect of Ivy, Stevia or Ivy+ Stevia on metabolic weight and N balance

All values are means of eight animals. $BW^{0.75}$; metabolic body weight.

	Treatment					
	Control	Ivy	Stevia	Ivy+ Stevia	SED	P
$BW^{0.75}$	25.29	25.23	25.51	25.45	0.178	0.371
N intake g/kg $BW^{0.75}$	0.507	0.508	0.502	0.502	0.003	0.236
Fecal N	0.271	0.259	0.253	0.247	0.013	0.375
Urine N	0.109	0.131	0.117	0.100	0.012	0.091
Digestible N	0.236	0.249	0.249	0.255	0.014	0.591
N retention	0.128	0.118	0.132	0.155	0.018	0.275
N utilization						
Digestible N/N intake	0.464	0.490	0.495	0.507	0.027	0.468
N retention/N digestible	0.540	0.473	0.529	0.603	0.052	0.141
N retention/N intake	0.251	0.233	0.263	0.307	0.036	0.244

3.3 Nutrient digestibility (NDF, ADF, and Ash)

The effect of control, Ivy, Stevia and Ivy+ Stevia diets on cannulated sheep has not shown any statistically significant effect (P>0.05) on nutrient digestibility, which measured by NDF, ADF and Ash techniques (**Table 3.3**). In the **Table 3.3**, dry matter (DM), organic matter (OM), crude protein (CP), NDF and ADF values have been shown for three different diets and control one. The samples were collected from 15th to 21st day of experiment to measure nutrient digestibility of animals.

Table 3.3. Effect of Ivy, Stevia or Ivy+ Stevia on apparent digestibility of nutrients

All values are means of eight animals.

	Treatment					
	Control	Ivy	Stevia	Ivy+ Stevia	SED	P
DM	0.564	0.581	0.573	0.591	0.020	0.556
OM	0.576	0.595	0.581	0.603	0.018	0.448
CP	0.464	0.490	0.495	0.507	0.027	0.468
NDF	0.540	0.556	0.543	0.563	0.022	0.714
ADF	0.439	0.479	0.444	0.457	0.033	0.633

3.4 Methane production

Supplementation of Ivy and Stevia had no effect on releases of methane (**Table 3.4**). A combination of Ivy with Stevia also had no effect on animal methane emission ($P>0.05$). Methane emission measurements have been recorded during last five days of animals when been kept in individual metabolic crates where placed in the methane chambers. In the **Table 3.4** besides methane emission gram per day (CH_4 g/day); methane emission for liter per day (CH_4 l/day) and also for emission gram per kilogram of metabolic body weight (CH_4 g/kg$^{0.75}$) have been calculated.

Table 3.4. Effect of Ivy, Stevia or Ivy+ Stevia on methane production

	Treatment					
	Control	Ivy	Stevia	Ivy+ Stevia	SED	P
DMI (g/day)	1155	1155	1155	1155		
CH_4 (g/day)	20.46	21.12	20.06	19.21	1.125	0.415
CH_4 (l/day)	28.65	29.57	28.09	26.89	1.575	0.415
CH_4 ($g/kg^{0.75}$)	0.813	0.843	0.787	0.76	0.044	0.31

4. DISCUSSION

In the recent study, neither Ivy and Stevia diets alone nor Ivy+ Stevia diet combination have not showed any significances (P>0.05) for cannulated sheep when compared to the control diet; regarding to the apparent digestibility of nutrients, metabolic weight, N balance and methane production.

4.1 Nutrient digestibility (DM, OM, CP, NDF and ADF)

For looking apparent nutrient digestibility DM, OM, CP, NDF and ADF measurements have been recorded. In the present study; there were no significant effects of administration of Ivy, with or without Stevia diets on apparent nutrient digestibility of DM, OM, CP, NDF, and ADF (P>0.05).

Although, the measurements of DM have not differentiated significantly between control sheep and treated one (Ivy, Stevia or Ivy+ Stevia), ranged from 0.564 (for control) to 0.591 (for Ivy+ Stevia; see Table 3.3) and the other treatments (Ivy, Stevia) between this range.

The values measured for OM was 0.576 in control and 0.603 for Ivy+ Stevia and the treatments (Ivy, Stevia) were between this range. Pen *et al.* (2007) OM was 0.686 for control and 0.701 for treated one (*Q. saponaria* extract). CP, NDF and ADF values also were measured respectively as follows for control sheep and treated sheep (Ivy+ Stevia); CP: 0.464 and 0.507, NDF: 0.540 and 0.563, ADF: 0.439 and 0.457. None of these measurements have shown any significant differences (P>0.05) among control and treated sheep (Ivy, Stevia, and Ivy+ Stevia).

A number of studies with saponins in sheep (Pen *et al*., 2007; Klita *et al*., 1996) also showed no differences in nutrient digestibility compared with non-supplemented animals. As adifferent ruminant study, cows, Holtshausen *et al*. (2009) in their trial feeding saponin (10 g/kg DM; *Y. schidigera* and *Q. saponaria*), did not affect methane emission, rumen fermentation and nutrient digestibility (DM, CP, NDF, ADF and gross energy).

Pen *et al.* (2007) measured DM for control 0.669 and treated one (*Q. saponaria* extract) as 0.683. Klita *et al.* (1996) have used alfalfa root -as a saponin source- in variety doses (0, 1, 2 and 4 % of DMI) in their trial to study the effect of saponins on digestive functions of sheep. They have applied a diet values ranging as follows for DM, OM, NDF and ADF respectively, which has not showed any significant effect (P>0.05); 1.185-1.117, 1.071-1.010, 668-631 and 402-380 (g/d). Pen *et al.* (2007) have not recorded any significant effect on digestibility nor A/ P ratio and protozoa in the rumen environment.

Saponins are believed to alter the digestion and utilization of dietary nitrogenous compounds in sheep. It is also believed that adaptation of the mixed ruminal microorganisms to saponins may be a main factor contributing to variability of antiprotozoal activity of saponin containing plants (Wallace *et a*l., 2002).

4.2 Chemical composition of hay and sugar beet

In the present trial 10 g/day of Ivy and 1.2/0.25 kg/day of ryegrass hay/sugar beet has been given to the sheep for 21 days. The chemical composition of DM (dry matter) was 795 and 807 g/kg of fresh DM; OM (organic matter) was 932 and 878 g/kg of DM; CP (crude protein) was 62.7 and

99.8 g/kg of DM; NDF (neutral detergent fibre) was 683 and 393 g/kg of DM; and ADF (acid detergent fibre) was 367 and 171 g/kg of DM for hay and sugar beet respectively (see Table 3.1).

Pen *et al.* (2007) have had a trial using sheep for period of 18 days by using saponin source (*Q. saponaria*, 13.5 g/kg DM or 16.1 g/day) and with ryegrass hay/concentrate (60/40) ratio. They have measured the chemical composition (g/kg) for Italian ryegrass hay and concentrate diet as follows respectively; DM 907.6 and 875.9, OM 938.5 and 929.1; CP 61.5 and 213.8; NDF 649.8 and 178.9; ADF 376.6 and 76.4; which have a similar result pattern as present study.

4.3 Effect of diets on metabolic weight and N balance

In the present trial, neither saponins (Ivy) nor saponins + glycosidase inhibitor (Ivy+ Stevia) extracts have a significant effect on cannulated sheep metabolic body weight, N intake, excretion of N in faeces and urine and N retention. Values have ranged between 17.10 - 17.03 for N intake, 9.14 - 8.38 for N excretion in faeces, 3.68 - 3.39 for N excretion in urine and 4.32 - 5.26 for N retention, as control and Ivy+ Stevia respectively. There were no effect of administration of saponins with or without Stevia on N utilization values (**Table 3.2**).

Comparing to these values Pen *et al.* (2007) also measured similar pattern, which agrees to the present results, for the effect of saponin containing diet on N intake, excretion of N in faeces and urine, and N retention. On the contrary, Klita *et al*. (1996) measured the increased flows of OM, NDF and N to the duodenum (g/day), with saponin treatments to sheep; but the decreased values for apparent total tract digestion and apparent ruminal digestion (%).

4.4 Methane production

Ivy, Stevia and Ivy+ Stevia diets have not shown any significant changes (P>0.05) comparing with control diets (see Table 3.4). The methane release per day was measured as 28.65 l/day for control group and 26.89 l/day for Ivy+ Stevia sheep.

Klita *et al.* (1996) in their trial methane release per day as follows for doses of 0, 1, 2 and 4 % of DMI respectively; 28.7, 30.05, 30.2 and 28.2 (L/d). The significant level also were low; for linear P=0.83 and quadratic effect P=0.57. In their study 100 % forage diet have been used while ryegrass hay (1.2 kg) and sugar beet (250 g) used in the present study. The potential effect of dietary strategies (such as; changing forage species or forage quality and improving concentrate: forage ratio) rather than additives has been reviewed in various studies (Beauchemin *et al*., 2008; Eckard *et al*., 2010; Martin *et al*., 2010)

Pen *et al.* (2007) have measured methane release as a 38.84 l/day for control and 32.55 for saponin extract fed sheep. Holtshausen *et al*. (2009) showed insignificant effect of saponins on early lactating cows' methane emission. Since passage rate and methane production is inversely related (Okine *et al*., 1989), it has been hypothesized that methane production would be increased in response to a decreased passage rate by feeding sheep with saponin extracts (Klita *et al*., 1996).

On the contrary, tea saponin (3 g/d) in lambs significantly decreased the methane emission compared to the control diet (Mao *et al*. 2010). Parallel to the latter study, *in vitro* studies also

showed a decreased methane emission by feeding animal with saponin rich sources (Jayanegara *et al*., 2014).

5. CONCLUSIONS AND RECOMMENDATIONS

Saponin containing plants or extracts seem to have the potential to act as a natural rumen manipulator. Saponins' main effect is to modify the composition of microbial populations (microbiological effect) which leads to the manipulation of rumen fermentation. The crucial microbiological effect of saponins in the rumen is the inhibition of ciliate protozoa, consequently and indirectly, improving the efficiency of microbial protein synthesis due to the decreased microbial protein turnover and protein flow to the duodenum.

Another desired effect of saponins is the inhibition of methane production through defaunation of ruminant directly by decreasing the activities of methanogens. In this regard saponins may decrease the rate of methanogenesis or expression of methane producing genes. Saponins have been used selectively to alter specific bacteria and fungi species, aiming to alter rumen metabolism beneficially or adversely.

Besides their primary effects, saponins also have physiological effects and have been implicated to alter rumen metabolism, such as ammonia adsorption and modulation of digesta passage rate in the rumen, but this physiological effects of saponins are generally negligible comparing to microbiological effects.

All microbiological and physiological effects and mechanisms of saponins over the rumen microbial population and rumen fermentation are interdependent depending on the concentrate and saponins type, diet composition, microbial populations have been affected and their adaptation capabilities to the saponins.

Further research is a need to understand different interactions of saponins' chemical structures and also nutrient composition of different diets and their effects over the rumen microbial populations. It is crucial to detect the most bioactive saponins against protozoa or their activities, and indirectly stimulate the number of methanogens; bacteria and fungi.

Although saponins antiprotozoal effect is transient the understanding long term rumen microbial adaptation mechanism which is lessening the antiprotozoal effect of saponins, may overcome this problem. The mechanism thought to decrease the antiprotozoal effect of saponins is increased activity of glycosidase in rumen microbes therefore, the inhibitor of glycosidase enzyme such as DMDP may be a solution in this regard.

In spite of being safe when administered orally, certain kinds of saponins may show toxic effects. So it should be tested *in vivo* in long term experiments.

Saponins can be applied in various feeding systems due to their beneficial effects both microbiological and physiological, if their proper active metabolites easily and cost effectively being isolated and identified from their plant sources. Biotechnological tools can be applied to improve the effective target-bioactive saponins.

The transitory effect of saponins have been reported previously due to the cleavage of the glycosidic bond by rumen microbes (Newbold *et al.,* 1997). The aim of this study is not just to examine the effect of saponin containing diets but also to apply a combination of saponin containing diet with a common natural glycosidase inhibitor (2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine, DMDP) containing Stevia extracts. Therefore, it has been hypothesized that

the combination of saponin containing diet with glycosidase inhibitors would avoid de-glycosylation, maintaining the intact saponin and so the activity in the rumen microbiota. The previous studies have recorded the greater effect on the fermentation pattern and protozoa motility when a combination of an ivy fruit extract rich in saponins (added at 1 mg/ml) with a glycosidase inhibitor DMDP (also added at 1 mg/ml) has been applied instead of saponin extract alone (unpublished data, IBERS).

To conclude the experiment results of present study, saponin containing diets (Ivy, Stevia and Ivy+ Stevia) results have not indicated any effect on cannulated sheep regarding to apparent digestibility of nutrients, metabolic weight and N balance and neither methane production. However, further study should be done to analyze protozoa count and also bacteria diversity to find out in which level the protozoa has been affected by Ivy, Stevia and Ivy+ Stevia containing diets and if the results in this study is correct, which bacteria species probably have been engulfed by protozoa and their symbiotic relations with methanogens

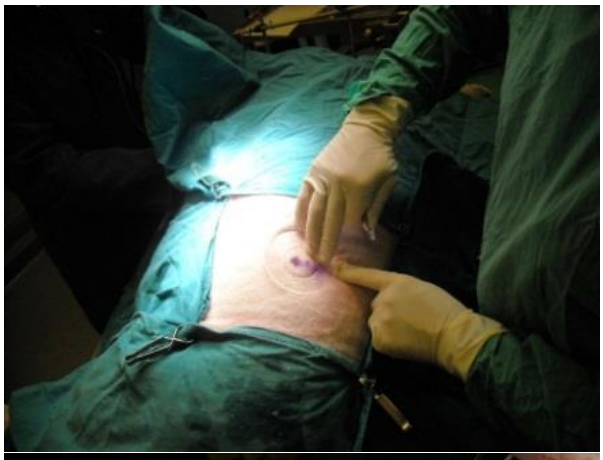
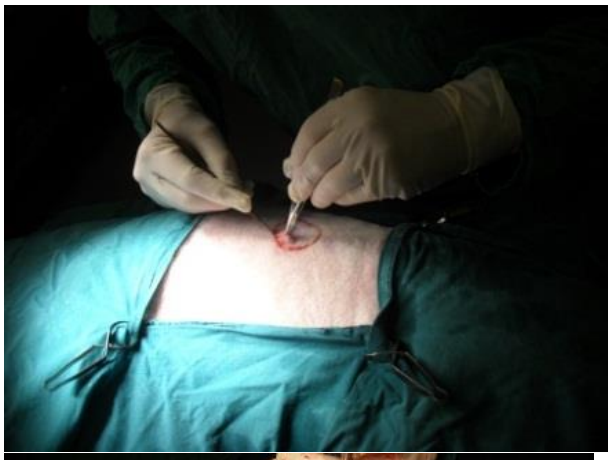
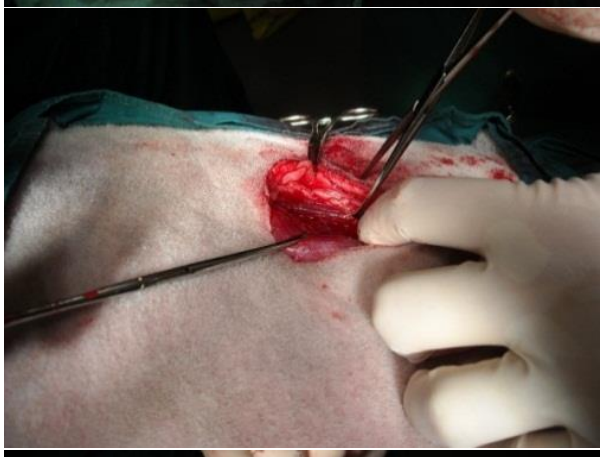
6. APPENDICES

plant	experiment	dosage	substrate/feed	protozoa[a]
A. auriculuformis	in vitro	1.2 mg/mL	hay or hay/concentrate	–46 to –63
C. sinensis	in vitro	0.4–1.2%	corn meal/grass meal	–43 to –73
E. cyclocarpum fruits	in vitro	100 mg/g	grass hay:barley straw *Arachis pintoi*	+54
E. cyclocarpum leaves	in vitro	1–10%	lucerne	–100
	in vitro	10 mg/L	no substrate	–91 (A)
	in vitro	0.5–10 mg/mL	no substrate	–20 to –95 (A
	in vivo (cattle)	200 g/day	*Pennisteum clandestinum* and rice polishing	–25[c]
	in vivo (buffalo)	375 g/day	native grasses	–100[c]
	in vivo (sheep)	25–75 g/day	oaten chaff + 1% urea + lupin	no effect
	in vivo (sheep)	100 and 300 g/day	*Pennisetum* hay	+5
	in vivo (sheep)	200 g/day	barley silage–barley grain–soybean meal	–35
	in vivo (sheep)	200 g/day	barley grain–barley grain–soybean meal	decrease[c]
Enterolobium timbouva leaves	in vitro	1–10%	lucerne	–100
M. sativa	in vitro	0.5–4%	cellulose and starch	ND
	in vivo (sheep)	2–4%	concentrate:roughage	–34 and –66
P. dodecandra fruits	in vitro	10 mg/L	no substrate	–85 (A)
P. saman fruits	in vitro	100 mg/g	grass hay:barley straw *Arachis pintoi*	+54
Q. saponaria	in vitro	1.2 mg/mL	hay or hay/concentrate	–38 to –54
Q. saponaria	in vitro	0.1–0.4%	casein	–8
S. saman leaves	in vitro	10 mg/L	no substrate	–85 (A)
	in vitro	0.25–4 mg/mL	*Pennisetum* hay–	–11 to –49

Appendix 1 Effect of saponin containing plants on protozoa in rumen in vitro and in vivo (Wina *et al*., 2005).

Methane (g/animal per day)						
With protozoa	Defaunated	Response (% change)	Animal sp.	Diet	Notes	Reference
13.4	14.9	11.1	Sheep	Dried lucerne	Defaunated using a detergent, methane measured in a chamber after 10 weeks of defaunation	Bird *et al.* (2008)
13.9	15.1	8.6			25 weeks after defaunation	
15.7	14.0	−10.8	Sheep	Cellulose based	Defaunated using a detergent, methane measured in a chamber	Kreuzer *et al.* (1986)
8.2	9.1	11.1		Native starch		
12.0	5.7	−47.5		Steamflaked starch based		
13.4	9.4	−29.9**	Sheep	Roughage and concentrate (3 : 7)	Defaunated using a detergent, methane measured in a chamber	Chandramoni *et al.* (2002)
12.6	13.1	4.0	Sheep	Roughage and concentrate (2 : 8)	Defaunated using a detergent, methane measured in a chamber	Hegarty *et al.* (2008)
15.6	16.1	3.2		Roughage		
25.1	18.6	−25.9*	Sheep	Roughage and concentrate (1 : 1)	Isolated at birth, methane measured in a chamber	Yanez-Ruiz *et al.* (2007)
31.5	23.9	−24.1*	Sheep	Roughage and concentrate (3 : 1)	Defaunated by rumen washing, methane measured using SF6.	Morgavi *et al.* (2008)
129.6	64.1	−49.6**	Cattle	Barley with a protein supplement	Defaunated using a detergent, methane measured in a chamber	Whitelaw *et al.* (1984)
16.6	18.2	9.6	Sheep	Maize silage concentrate diet supplemented with protected fat	Defaunated using a detergent, methane measured in a chamber	Machmüller *et al.* (2003
14.3	14.6	2.1		Supplemented with coconut oil		
14.1	14.6	3.5	Goat	Hay	Isolated at birth methane measured in a chamber	Itabashi *et al.* (1984)
22.9	17.9	−21.8**		Hay plus concentrate		
18.6	16.4	−11.8		Hay plus concentrate plus monensin		
		−10.5				Average response

Appendix 2. The effect of defaunation on methane production as measured in in vivo experiments (Morgavi *et al.*, 2010)


Appendix 3. Sheep cannulation surgery process held on 9-11 December 2014

7. REFERENCES

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